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The Oral Commensal, *Streptococcus gordonii*, synergizes with Tat protein to induce HIV-1 promoter activation in Monocytes/Macrophages

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Abstract

Trans-activator of Transcription (Tat) is an HIV-1 protein essential for viral replication. Oral periodontopathogens (e.g., *Fusobacterium nucleatum*) enhance HIV-1LTR promoter activation in monocytes/macrophages in absence of Tat; however, some oral commensals fail to trigger this response. We sought to determine the effect of Tat on HIV-1LTR promoter activation induced by the representative oral commensal *Streptococcus gordonii* in monocytes/macrophages. *S. gordonii* enhanced HIV-1LTR reactivation in THP89GFP (Tat⁺), but not in BF24 (Tat⁻) cells. Interestingly, *S. gordonii*, but not *S. sanguinis* enhanced HIV-1LTR activation in the presence of recombinant Tat in BF24 cells. This response correlated with IL-8 but not TNF α or IL-6 production, and was abrogated by the NF κ B inhibitor BAY 11-7082. Kinetics of NF κ B-RelA activation did not explain the *S. gordonii*-induced HIV-1LTR activation in presence of Tat. These results suggest that *S. gordonii*-induced HIV-1 reactivation in monocytes/macrophages is Tat-dependent and appears to involve NF κ B activation.

Keywords

HIV-1; *S. gordonii*; monocytes/macrophages; periodontitis; Transcriptional *Trans*-activator; oral bacteria

1. INTRODUCTION

The ability of HIV-1 to become integrated into the genome of host cells (i.e. T cells, monocytes/macrophages and dendritic cells) that survive the viral infection is one of the main obstacles to complete eradication of this infectious disease, since these HIV-1 cell reservoirs are refractory to Highly Active Antiretroviral Therapy and a critical source for viral recrudescence. [1-3] The mechanisms associated with HIV-1 latency and reactivation are not fully understood; however evidence suggests that due to the presence of binding sequences upstream of the HIV-1LTR promoter for host cell transcription factors (e.g. NF κ B, Sp1, C/EBP, etc), reactivation of the integrated HIV-1 genome (i.e. HIV-1 provirus) in latently infected cells involves not only viral, but also cellular transcriptional regulators, in order to produce viral transcripts and further assemble of new virions.[4, 5] Among the HIV-1 regulatory proteins, Tat plays a critical role in HIV-1LTR activation as a positive regulator enhancing the expression of new HIV-1 transcripts.[6, 7] It is generally accepted that Tat promotes the assembly of an active transcription elongation complex that involves interaction between host transcription factors (e.g. NF κ B, Sp1) bound to the upstream

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control region and other transcription factors such as TATA binding protein (TBP), transcription factor IIH (TFIIH), P-TEFb (transcription elongation factor composed of cyclin TI and cdk9) and RNA polymerase (Pol) II.[7] It has been shown by *in vitro* studies that Tat is not packaged into virions, but it is normally released by HIV-1 acutely infected cells compromising neither cell viability nor cell permeability.[8, 9] In fact, plasma levels of Tat in HIV-1⁺ patients ranging from 2-40 ng/ml have been reported; however this level could be underestimated due to the ability of Tat to be trapped by extracellular matrix molecules.[10, 11] Extracellular Tat can act both from outside and inside the cells depending on its concentrations and the target cell type. Thus, lower concentrations (picomolar) appear to act through membrane receptors and activation of signal transduction pathways (e.g. integrin receptors, vascular endothelial growth factor receptor 2-VEGFR2).[12-14] In contrast, nanomolar and micromolar Tat concentrations are generally internalized and rapidly translocated (about 30 minutes) into the nucleus by several cell types including monocytes and macrophages.[9, 15] In addition to enhancing HIV-1 promoter activation, Tat has exhibited the ability to modulate the expression of several cellular genes including cytokines/chemokines (e.g. up-regulation of IL-8, IL-6, IL-10 and down-regulation of IL-12). The diverse number of effects in host cell gene expression associated with Tat is consistent with events associated with AIDS progression, as well as disorders commonly found in HIV-1⁺ patients.[6, 16]

We have previously shown that oral bacteria related to local (i.e. periodontal disease) and systemic inflammatory disorders differentially enhance HIV-1LTR promoter activation in T cells, monocytes/macrophages and dendritic cells, [17] and HIV-1 recrudescence induced by *P. gingivalis* and *F. nucleatum* in monocytes/macrophages involves Toll like receptor-2 (TLR2) and TLR9 activation, as well as host cell transcription factors such as NFκB and Sp1.[18] An interesting observation from those studies was that oral periodontopathogens induced a significant HIV-1LTR promoter activation in the BF24 monocytes/macrophages model, which harbors the HIV-1LTR promoter, but not critical HIV-1 transcriptional regulators such as Tat. In addition, we were not able to detect HIV-1LTR promoter activation in BF24 cells exposed to the oral commensals *S. gordonii* and *S. sanguinis*. Based on these results, and the role of Tat as a positive regulator in HIV-1LTR activation, we hypothesized that HIV-1 promoter activation induced by these oral commensals could be Tat-dependent. To address this question we evaluated the HIV-1LTR promoter activation in BF24 monocytes/macrophages induced by these representative oral commensal bacteria in the presence or absence of a recombinant form of Tat. Furthermore, potential mechanisms involved in Tat-dependent HIV-1LTR promoter activation were evaluated.

2. MATERIAL AND METHODS

2.1. Bacterial strains and growth conditions

The bacterial strains used in these studies were *Porphyromonas gingivalis* (ATCC 33277) grown in anaerobic broth (Becton Dickinson, Sparks, MD, USA) supplemented with 5μg/ml Hemin and 1μg/ml Menadione. *Fusobacterium nucleatum* (ATCC 25586), *Streptococcus gordonii* (ATCC 10558), and *Streptococcus sanguinis* (ATCC 10556) grown in Trypticase soy broth supplemented with 0.6% yeast extract (TSBYE) (Becton Dickinson, Sparks, MD, USA). All bacterial cultures were grown at 37°C under anaerobic conditions (80% N₂, 10% H₂, and 10 % CO₂) or aerobic conditions for *S. sanguinis* as described previously.[19-21] Bacterial extracts from each microorganism were obtained as we have previously reported. [22, 23] Briefly, 5 colonies grown on blood agar plates were placed into 25 ml of corresponding broth and incubated for 24h. Then, bacterial cultures were transferred into 500 ml of the same broth, and incubated under the same conditions for 24h. The bacterial suspension was washed three times with sterile phosphate-buffered saline (PBS) at 10,000 g for 20 minutes at 4°C. The pellet was resuspended in 15 ml PBS with complete EDTA-free

protease inhibitor cocktail (Roche, Mannheim, Germany), followed by sonication using an ultrasonic disrupter (Branson Digital Sonifier model 450, Danbury, CT). Disruption of bacteria was confirmed by light microscopy. The crude extract after sonication was centrifuged at 13,000 g for 10 minutes at 4°C and supernatants were evaluated for protein concentration by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL), and used to challenge cells concentrations between 1 and 10 µg/ml of protein.

2.2. Cell cultures

BF24 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Barbara K. Felber and Dr. George N. Pavakis. [24] The BF24 cell line is a subclone of the monocytic leukemia cell line THP-1 that was stably transfected with the HIV-1LTR promoter driving the CAT reporter gene expression. THP89GFP cells were a generous gift of Dr. David Levy (NYU) and express enhanced green fluorescent protein (EGFP) under the control of the HIV-1 promoter without removing any viral sequences. Fluorescence and virus production are tightly coupled in THP89GFP cells.[25] Both cell types were cultured in RPMI 1640 with L-glutamine and 10% Fetal Bovine Serum and maintained in a 5% CO₂ atmosphere at 37°C. In addition, 100 U/ml penicillin and 100 µg/ml streptomycin were used for THP89GFP cultures.

2.3. Stimulation of monocytes/macrophages with bacterial extracts and rTat

The recombinant form of Tat (Cat # 2222) was obtained through the NIH AIDS Research Reagent Program, Division AIDS, NIAID, NIH from Dr. John Brady.[26] BF24 cells were seeded in 24-well plates at a cell density of 2.5×10^5 cells/well in 1ml of RPMI1640 medium supplemented with 2% FBS and pre-incubated or not for 30 minutes with several concentrations of rTat reconstituted in buffer (0.1% BSA in PBS) immediately prior to being used as previously shown.[9] Further, when indicated different concentrations of each bacterial extract were added and cells incubated 16h more before analysis.

2.4. ELISAs

After several treatments, HIV-1LTR/CAT activity in BF24 cell lysates and cytokine/chemokine (i.e., IL-8, TNFα, and IL-6) levels in supernatants were determined by sandwich ELISA (eBioscience, San Diego, CA; R&D Systems, Minneapolis, MN; Roche, Mannheim, Germany), as we previously described [22]. Viral production was determined by measuring p24 levels in supernatants from THP89GFP cells by ELISA (Advanced BioScience Laboratories, Inc. Kensington, MD). For each case, absorbance was measured using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) at the indicated wavelengths and levels determined from standard curves for each molecule.

2.5. Fluorometry

Experiments involving THP89GFP cells were performed and HIV-1/EGFP activity was determined by fluorometry. Briefly, after different treatments, THP89GFP cells were harvested and washed in PBS at 1,300 X g for 5 minutes. Pellets were resuspended in 150 µl of lyses buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40) for 20 minutes on ice, and cell lysates were then centrifuged at 10,000 g X for 5 minutes at 4°C. Further, 100 µl of supernatants were added into *Optilux* 96-well Clear-Bottom Plates (BD Falcon, Franklin Lakes, NJ) for fluorescence analysis using the Microplate Fluorescence Reader FLx800 (BIO-TEK Instruments, Inc., Winooski, Vermont). The wavelengths used for excitation and emission were 485nm and 528nm respectively.

2.6. Effect of NFκB inhibitor BAY 11-7082

To determine the potential role of NFκB activation in HIV-1LTR promoter activation induced by *S. gordonii* in the presence of rTat, BF24 cells pre-treated with 100 ng/ml rTat for 30 minutes were stimulated with 10 μg/ml of *S. gordonii* extract either in presence or absence of different concentrations of the specific NFκB inhibitor, BAY 11-7082 (Calbiochem, San Diego, CA). Subsequently, cell lysates and supernatants were analyzed for HIV-1LTR/CAT activity and IL-8 production, respectively by ELISA (R&D Systems, Minneapolis, MN, USA).

2.7. Nuclear extracts preparations

Nuclear extracts were prepared using a nuclear extract kit (Active motif, Carlsbad, CA). Briefly, 2.5×10^5 cells/well were seeded in 24-well plates with 0.5 ml of RPMI 1640 supplemented with 2% FBS. After different treatments cells were harvested and washed at 20 X g for 5 minutes at 4°C. Then pellets were washed once more with 1 ml ice-cold PBS containing phosphatase inhibitors and pellets resuspended in 250 μl 1X hypotonic buffer followed by incubation on ice for 15 minutes. After that, 25 μl of detergent were added and tubes were vortexed 10 seconds. The suspension was centrifuged for 30 seconds at 14,000 x g at 4°C, and the pellet (nuclear fraction) was resuspended in complete lyses buffer followed by vortexing for 10 seconds. The suspension was incubated on ice for 30 minutes on a rocking platform followed by vortexing for 30 seconds and centrifugation for 10 minutes at 14,000 x g at 4°C. Supernatants containing the nuclear fraction were transferred into pre-chilled tubes and stored at -80°C until use.

2.8. TransAM transcription factor ELISA

For kinetic analyses of NFκB activation, levels of NFκB-RelA (p65) were quantified in nuclear extracts from BF24 cells after different treatments using an ELISA-based TransAM™ kit (Active motif, Carlsbad, CA, USA). Briefly, 30 μl of complete binding buffer were added into 96-well plate with an immobilized oligonucleotide containing an NFκB consensus binding site (5' – GGGACTTTCC – 3'). Then, 20 μl of nuclear extract or standards (recombinant RelA) diluted in lyses buffer were added in duplicate and plates incubated 1h at room temperature (RT). After washing, the plates were incubated first for 1h at RT with 100 μl/well primary anti-RelA followed by incubation with a secondary antibody conjugated with horseradish peroxidase. Finally after adding the substrate; absorbance was measured using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

2.9. Statistical Analysis

The significance of any difference between the means for the experimental groups was determined by a Kruskal Wallis ANOVA with Dunnett's test for multiple comparisons and the Mann-Whitney U test (SigmaStat 3.5; Systat Software, Point Richmond, CA). The values were considered significantly different if P was ≤ 0.01.

3. RESULTS

3.1. *S. gordonii* induces HIV-1LTR promoter activation in THP89GFP cells but not in BF24 cells

The promoter activation in BF24 cells, containing the HIV-1 promoter without viral proteins, and HIV-1 latently infected THP89GFP cells (*i.e.* expressing Tat), was compared at different time points. As observed previously, the *S. gordonii* extract was not able to enhance HIV-1 promoter activity in BF24 cells, although it was increased by the same concentration of the *P. gingivalis* extract (Fig. 1A). As predicted, the same *S. gordonii*

extract concentration induced HIV-1/EGFP promoter activity, as well as HIV-1 production determined by p24 levels in THP89GFP cells at 48 and 72 hours post-challenge (Fig. 1B & C). These results suggest that HIV-1 viral proteins involved in HIV-1 promoter activation, such as Tat, could be critical for HIV-1 promoter activation in monocytes/macrophages leading to viral reactivation induced by *S. gordonii*.

3.2. Exogenous rTat HIV-1 protein synergizes with *S. gordonii* to induce HIV-1 promoter activation and IL-8 production in BF24 monocytes/macrophages

The ability of two representative oral commensal bacteria (*S. gordonii* and *S. sanguinis*) to trigger HIV-1 promoter activation in BF24 monocytes/macrophages was first evaluated. Although BF24 cells were activated by both oral commensals as determined by dose-dependent IL-8 production (Fig. 2A), HIV-1LTR CAT activity was not enhanced by neither *S. gordonii* nor *S. sanguinis* (Fig. 2B). Importantly, IL-8 levels induced by *S. gordonii* were approximately double the IL-8 levels induced by *S. sanguinis* when cells were exposed to the higher bacterial extract concentrations (10 µg/ml). Further, the biological activity of rTat was first confirmed by determination of IL-8 levels in supernatants from BF24 cells exposed to different rTat concentrations as previously shown.[27] When BF24 cells were exposed to concentrations of rTat greater than 10 ng/ml, IL-8 production was increased by 5-24 fold (Fig. 3A). Similar concentrations of rTat exhibited the ability to significantly increase HIV-1 promoter activity particularly with higher rTat concentrations (100-500 ng/ml) (Fig. 3B). Subsequently, the ability of *S. gordonii* and *S. sanguinis* to enhance HIV-1 promoter activity in the presence of Tat was tested using an rTat concentration (100 ng/ml) that is biologically active and within the range of plasma levels previously reported in HIV-1⁺ patients.[10] Interestingly, *S. gordonii*, but not *S. sanguinis* extract significantly increased the HIV-1 promoter activation in the presence of rTat. As noted in previous results none of these extracts triggered HIV-1 promoter activation in cells incubated only with media compared to unstimulated cells (Fig. 3C & D). When BF24 cells were exposed only to *S. gordonii* and *S. sanguinis* extracts, IL-8 production was significantly up-regulated as expected (Fig. 3E & F). Nevertheless, although this response induced by both oral commensals was increased by the presence of rTat, cells that were challenged with *S. gordonii* extract showed a notable synergistic effect in IL-8 production (Fig. 3E). Similar treatments of BF24 cells with *S. gordonii* extract or rTat individually or in combination did not enhance the production of pro-inflammatory cytokines TNFα or IL-6 as it was indeed observed in cells exposed to bacterial extracts from Gram-negative periodontopathogens (Fig. 4A & B).

3.3. Kinetics of NFκB activation does not explain HIV-1LTR promoter activation induced by *S. gordonii* in the presence of rTat

It has been shown that IL-8 production and HIV-1 replication are mostly regulated at the transcriptional level and as a common characteristic; both IL-8 and HIV/LTR promoters have consensus binding sequences for NFκB.[5, 28, 29] A positive correlation between IL-8 and HIV-1 promoter activation was observed when BF24 cells were challenged with *S. gordonii* in the presence of rTat. Therefore, it was likely that NFκB activation could be involved in this response. As expected, the specific NFκB inhibitor BAY 11-7082 totally abrogated the HIV-1 promoter activity induced by *S. gordonii* in the presence of rTat in B24 cells (Fig. 5A); nonetheless, IL-8 levels in the supernatants induced by *S. gordonii* alone or in presence of rTat were unaffected by the same NFκB inhibitor concentrations (Fig. 5B).

An intriguing result was that in spite of the cellular activation induced by *S. gordonii*, which is reflected by IL-8 production in BF24 cells, there was no an increase in HIV-1 promoter activity as was indeed previously observed after challenge with selected periodontal pathogens or with *S. gordonii* in the presence of rTat. Thus, activation of transcription factors related to cytokine/chemokine production (e.g. NFκB) were not sufficient to trigger

viral reactivation and perhaps other processes related to the kinetics of NFκB activation could be involved. To address this hypothesis nuclear extracts from BF24 cells exposed to either *F. nucleatum* or *S. gordonii* were evaluated for the levels of activated NFκB/RelA (p65) at different time points. As was anticipated, although increased RelA levels were detected in BF24 cells challenged with both bacterial extracts during the first 30 min. and 1 hour, there was about 25-fold higher levels of activated RelA in cells exposed to *F. nucleatum* compared with the response induced by *S. gordonii* (Fig. 5C). After the first hour post-bacterial challenge, only BF24 cells challenged with *F. nucleatum*, but not *S. gordonii*, had increased levels of activated RelA (Fig. 5C). In a similar manner, the potential variations in the kinetics of RelA activation were evaluated in BF24 cells exposed to *S. gordonii* in the presence or absence of rTat. As expected, BF24 cells exposed to rTat exhibited higher and prolonged levels (*i.e.* until 6h post-challenge) of RelA activation compared with the effect of *S. gordonii* alone. Interestingly, the rapid and higher RelA activation induced by rTat showed a decrease in the first hour, followed by a further increase at 3 and 6 hours. Nevertheless, there were no significant differences in the kinetics of NFκB(RelA) activation when cells were challenged with *S. gordonii* in the presence of rTat when compared with the effect elicited by rTat alone (Fig. 5C).

4. DISCUSSION

Chronic immune activation associated with the presence of bacterial components (*i.e.* LPS, DNA) in the blood of HIV-1⁺ patients has been linked to HIV-1 recrudescence and AIDS progression.[30, 31] We and others have shown that activation of transcription factors, such as NFκB upon Toll-like receptor engagement by bacteria, appears to play a critical role in pathogen-induced HIV-1 promoter activation from latently infected cells.[23, 32] Oral commensals are the predominant species at the oral mucosa and can be similarly recognized by TLRs enhancing activation of host cell transcription factors involved in HIV-1LTR positive regulation such as NFκB.[33, 34] Whether or not these bacteria induce HIV-1LTR activation remains to be determined. In contrast to what we previously observed for periodontal pathogens[22], the representative oral commensal *S. gordonii* failed to induce HIV-1 promoter activation in BF24 monocytes/macrophages but not in THP89GFP cells. The inability of this oral commensal to enhance HIV-1 promoter activation could be explained in part by the absence of the positive HIV-1 transcriptional regulator Tat in the BF24 cell model. Consistent with this hypothesis, HIV-1 promoter activation was synergistically enhanced by *S. gordonii*, but not *S. sanguinis* in the BF24 cells pre-incubated with rTat. Previous observations showing that pro-inflammatory cytokines produced in response to pathogenic bacteria are involved in HIV-1LTR promoter activation[35, 36], and the ability of Tat to induce cytokine/chemokine production in monocytes/macrophages including TNFα[37], led us to hypothesize that *S. gordonii*-induced HIV-1 promoter activation in the presence of rTat could involve pro-inflammatory cytokines. However, we were not able to detect TNFα or IL-6 in supernatants from BF24 cells exposed to *S. gordonii* extracts alone or in the presence of rTat. Although the ability for BF24 cells to produce these pro-inflammatory cytokines appeared not to be impaired, since challenge with *P. gingivalis* or *F. nucleatum* extracts induced its production, the inability of rTat to promote TNFα and IL-6 production in BF24 cells could involve phenotypic differences between this transfected cell line and its parental, THP-1 cell line, as well as primary monocytes which produced TNFα in response to similar Tat concentrations.[37, 38] Moreover, the pro-inflammatory activity of Tat is a topic of controversy since evidence also suggests anti-inflammatory effects of this HIV-1 protein (*e.g.* IL-10 production), which may be associated with the immunosuppression observed in AIDS.[39]

A synergistic effect in IL-8 production by BF24 cells exposed to *S. gordonii*, but not *S. sanguinis*, in the presence of rTat, confirms that Tat is likely to play an important role in the

induction of IL-8 as previously shown.[40] Although IL-8 levels induced by *S. sanguinis* appear to be increased by rTat, there was not an increase in HIV-1LTR promoter activity, which suggests that even though this microorganism belongs to the same *S. gordonii* genus, there could be important structural differences between these 2 species that may account for this difference in response. Consistent with this observation, it has been suggested that structural components from *S. gordonii* (i.e., lipoteichoic acid, proteoglycans, DNA) have a differential ability to activate antigen presenting cells and modulate cytokine production.[33, 41] It is not fully understood how host microbial recognition of commensals and pathogenic microbes through specialized receptors (e.g. TLRs) that converge on common signaling intermediates and transcription factors, such as NFκB, induce distinct transcriptional outputs; however, emerging evidence suggests a role for post-translational modifications (i.e. phosphorylation and acetylation) of NFκB, as well as interaction of NFκB with co-operating transcription factors (e.g. AP-1, C/EBP, Sp1, STAT6, IRF3).[42]

Similar to HIV-1 promoter activation, IL-8 production appears to be mainly regulated at the transcriptional level and involves transcription factors, such as NFκB, AP-1 and C/EBPβ. [43] In addition, similar concentrations of rTat used in this study have shown the ability to activate NFκB in monocytes.[39] Thus, it was likely that the HIV-1 promoter activity induced by *S. gordonii* in the presence of rTat could involve transcriptional regulation by factors in common with the IL-8 and HIV-1LTR promoters, such as NFκB. Indeed, as seen previously for periodontal pathogens, HIV-1 promoter activation induced by *S. gordonii* in the presence of rTat was abrogated by the specific NFκB inhibitor BAY11-7082, suggesting a role for NFκB in this response. This effect appears to be specific, since IL-8 production induced by similar treatments was not affected by the same inhibitor concentrations.

An interesting observation was that in spite of the fact that IL-8 was induced by *S. gordonii* and rTat individually in BF24 cells, which indirectly would suggest a level of NFκB activation, HIV-1 promoter activity was significantly increased only by rTat treatment. These results suggest that the mere presence of activated NFκB in the nucleus could not be sufficient to trigger HIV-1 promoter activity in monocytes/macrophages. Previous studies in T cells have suggested that time and level of NFκB activation could be determinants of this response.[44], and the kinetics of NFκB activation appears to be critical in controlling the magnitude of TLR-mediated cytokine production.[45] Therefore, it would be expected that a higher and more prolonged NFκB activation after different treatments would correlate with successful HIV-1 promoter activation. Although this hypothesis was valid for the kinetic analysis of NFκB-RelA activation in BF24 cells exposed to *F. nucleatum* or rTat individually compared with the effect of *S. gordonii*, there were no changes in the kinetics of NFκB activation in cells exposed to both *S. gordonii* and rTat compared with the effect of rTat alone. It has been proposed that Tat may facilitate the association of RelA with other co-activators, such as CBP, and differentially induce binding of NFκB to different responsive promoters including IL-8 promoter.[27] Thus, it is likely that Tat-dependent HIV-1 promoter activation induced by *S. gordonii* could involve prolonged binding of NFκB to the HIV-1 promoter, which would need to be documented. We cannot rule out the possibility that other transcription factors such as Sp1, could be playing a role in this response since Tat also has shown the ability to modulate their transcriptional activity.[46]

In general these results suggest that the oral commensal *S. gordonii* induces HIV-1 promoter reactivation in monocytes/macrophages in a mechanism that appears to involve the HIV-1 protein Tat and the cell transcription factor NFκB. Future and more detailed molecular studies exploring the potential differences in the transcriptional activity of RelA and other transcription factors associated to the HIV-1 promoter activity enhanced by *S. gordonii* and Tat are warranted. Our studies indicate that HIV-1 reactivation induced by oral bacteria in monocytes/macrophages appears to involve both Tat-independent (i.e. periodontopathogens)

and Tat-dependent mechanisms, which could be related with particular bacterial structures and their ability to differentially activate the cells. Finally, HIV-1 reactivation induced by *S. gordonii* in a Tat-dependent manner appears not to be associated with an inflammatory response (i.e. TNF α production) as it was indeed observed with the periodontal pathogens *F. nucleatum* and *P. gingivalis*. [23] Thus, it is tempting to speculate that even in the absence of inflammation there could be a probability for HIV-1 to be reactivated from latently infected monocytes/macrophages in particular microenvironments. These would be represented by a threshold of activation of specific host transcription factors (e.g. NF κ B) by oral commensal bacteria that may be directly modulated by viral proteins, such as Tat, or indirectly by altered expression and activation of pattern recognition receptors (e.g. TLRs) previously shown in HIV-1-infected cells. [47] Since *S. gordonii* is highly colonizing the oral and nasopharyngeal mucosa and it has been considered as a potential vector candidate for an HIV-1 vaccine [48, 49], whether or not the viral production induced through these mechanisms by the oral commensal *S. gordonii* in monocytes/macrophages has a clinical relevance will require further studies.

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FIGURE 1. *S. gordonii* enhances HIV-1LTR activation in THP89GFP, but not in BF24 monocytes/macrophages

2.5×10^5 cells/ml were challenged with 10 μ g/ml *S. gordonii* extract and (A) HIV-1/CAT activity in BF24 cell lysates was determined by CAT-ELISA, and (B) HIV-1/EGFP activity as well as (C) p24 levels were determined in THP89GFP cells by fluorometry and ELISA respectively at different time points until 72h as described in methods. BF24 cells challenged with 10 μ g/ml of *P. gingivalis* extract or incubated only with media (Mock) were used as positive and negative controls respectively for HIV-1/CAT activation. The figures are representative of at least 2 independent experiments by triplicate (n=6). Data are expressed as means $\pm$ standard deviations. The asterisk (*) denotes $P \leq 0.01$ compared to the results for the control (mock), as determined by a Kruskal Wallis ANOVA with Dunnett's test for multiple comparisons. ND = Not detected.

FIGURE 2. Oral commensals *S. gordonii* and *S. sanguinis* fail to induce HIV-1LTR promoter activation in B24 monocytes/macrophages

BF24 cells (2.5×10^5 /well) were seeded into 24-well plates with 1 ml of media and left either untreated or challenged with different concentrations of bacterial extracts from *S. gordonii* and *S. sanguinis* for 16h. (A) IL-8 levels in supernatants and (B) HIV-1/CAT activity in cell lysates were determined by ELISA as described in methods. BF24 cells exposed to sodium butyrate (NB) or incubated only with media (Mock) were used as positive and negative controls respectively for HIV-1 promoter activity. The figures are representative of at least 2 independent experiments in triplicate ($n=6$). Data are expressed as means $\pm$ standard deviations. *, $p \leq 0.01$ compared to the results for the control (mock), as determined by a Mann-Whitney U test on ranks. ND= Not detected.

FIGURE 3. *S. gordonii* synergizes with rTat to enhance HIV-1LTR promoter activation and IL-8 production

BF24 cells (2.5×10^5 /well) were first incubated only with media or in presence of several concentrations of rTat (10-500 ng/ml). (A) IL-8 levels in supernatants and (B) HIV-1/CAT promoter activity in cell lysates were determined 16h post-treatment as described in methods. Using the same cell density, BF24 cells were pre-incubated 30 minutes with either 100 ng/ml rTat or media followed by challenge with different concentrations of bacterial extracts from *S. gordonii* or *S. sanguinis* and HIV-1/CAT promoter activity in cell lysates (C and D) or IL-8 production in supernatants (E and F) were determined by ELISA as described in methods. The figures are representative of at least 2 independent experiments by triplicate (n=6). Data are expressed as means $\pm$ standard deviations. Values that were significantly different ($p \leq 0.01$) from mock/media (*) or with/without rTat (#) as determined by a Kruskal Wallis ANOVA with Dunnett's test for multiple comparisons (A and B) or a Mann-Whitney U test on ranks (C-F). ND= Not detected.

FIGURE 4. Pro-inflammatory cytokine production by B24 monocytes/macrophages in response to *S. gordonii* and rTat

BF24 cells (2.5×10^5 /well) were seeded in 24-well plates with 1 ml of media and cytokine levels were determined by ELISA in supernatants 16h after different treatments as described in methods. (A) Levels of IL-6, TNFα and IL-8 in supernatants from BF24 cells challenged with different concentrations of either rTat (10-500 ng/ml) or 10 μg/ml of *S. gordonii* (Sg) extract are shown. (B) Levels of pro-inflammatory cytokines TNFα and IL-6 induced by either 10 μg/ml of *S. gordonii* extract or 100 ng/ml of rTat individually or in combination were determined by ELISA. BF24 cells exposed to oral Gram-negative *P. gingivalis* (Pg) or *F. nucleatum* (Fn) extracts and cells incubated only with media were used as positive and negative controls respectively for cytokine production. The figures are representative of at least 2 independent experiments in triplicate (n=6). Data are expressed as means ± standard deviations. *, $p \leq 0.01$ compared to the results for the control (mock/media), as determined by a Kruskal Wallis ANOVA with Dunnett's test for multiple comparisons (rTat) or a Mann-Whitney U test ranks (Pg and Sg vs. media and Fn vs. media). ND= Not detected.

FIGURE 5. NFκB activation is involved in HIV-1LTR promoter activation induced by *S. gordonii* in presence of rTat

BF24 cells (2.5×10^5 /ml) pre-incubated 30 minutes 100 ng/ml rTat were challenged with 10 μ g/ml of bacterial extract from *S. gordonii* either in presence or absence of different concentrations of NFκB inhibitor BAY 11-7082 for 16h and (A) HIV-1/CAT promoter activity in cell lysates, or (B) IL-8 levels in supernatants were determined by ELISA as described in methods. BF24 cells incubated only with media (mock) were used as a negative control (C) Kinetics of NFκB-RelA(p65) activation was evaluated in nuclear extracts from BF24 cells (2.5×10^5 /ml) challenged with 10 μ g/ml of bacterial extract from *S. gordonii*, 100 ng/ml rTat, or both (Sg + rTat), and 10 μ g/ml *F. nucleatum* extract by TransAM as described in methods for the indicated times. The figures are representative of at least 2 independent experiments by triplicate (n=6). Data are expressed as means $\pm$ standard deviations. Values that were significantly different ($p \leq 0.01$) comparing presence of NFκB inhibitor BAY 11-7082 for HIV-1/CAT and IL-8 determined by a Mann-Whitney U test on ranks (A and B) are indicated by asterisks (*), or by Kruskal Wallis ANOVA with Dunnett's test for multiple comparisons (C) For NFκB/p65 levels the asterisks (*) denote significantly different ($p \leq 0.01$) from *S. gordonii* alone and pound sign (#) denotes significantly different ($p \leq 0.01$) from *S. gordonii*/*F. nucleatum* induced NFκB/p65 levels at 0 time.